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Shujun Feng[1], Wenjing Pan[1], Ye Jin[1],
Elena S. Kapora[2], Jianhua Zheng[1], Indira V. Sakhautdinova[2]

MIR-25 IS UP-REGULATED IN OVARIAN CANCER AND PROMOTES CELL PROLIFERATION AND MOTILITY

[1]Harbin Medical University, Harbin, China
[2]Bashkir State Medical University, Ufa, Russia

Ovarian cancer (OC) is a major cancer-related mortality among women. Recent studies showed that many microRNAs (miRNAs) were dysregulated and involved in tumorigenesis of OC. The present study investigated the role of miR-25 in the development and progression of OC. The expression of miR-25 was increased in OC tissues and cell lines. Inhibition of miR-25 remarkably suppressed proliferation, migration, and invasion of OC cells. Large tumor suppressor 2 (LATS2), a tumor suppressor, was confirmed to be a direct target of miR-25 in OC cells. Moreover, restoration of LATS2 significantly attenuated the oncogenic effects of miR-25. Together, our data suggest an oncogenic role of miR-25 in OC, and a potentially novel diagnostic and therapeutic target for OC treatment.

Key words: ovarian cancer; miR-25; proliferation; migration; invasion.

Ovarian cancer (OC) is one of the most frequent gynecologic malignancies in women [1]. Epithelial ovarian cancer (EOC) accounts for approximately 90% of ovarian cancer, including serous adenocarcinoma, endometrial adenocarcinoma, and clear cell carcinoma [2]. Despite advances in the diagnosis and chemotherapy of this cancer, the 5-year survive rate is still poor [3]. Therefore, it is urgent to explore novel diagnostic targets for EOC.

MicroRNAs (miRNAs) are small noncoding RNAs which bind to the 3′-untranslated region (3′-UTR) of target mRNAs and inhibit gene expression via cleaving target mRNA or repressing mRNA translation [4]. MiRNAs have been involved in a wide range of biological processes, including proliferation, differentiation, migration, invasion, and angiogenesis [5, 6]. Aberrantly altered expression of miRNAs have been found in several cancers including OC, and many miRNAs have been identified as prognostic markers for some cancers [7, 8]. In OC, miRNAs act as either oncogenes or tumor suppressor [9,10]. MiR-25 has been found to be increased in OC, however, its detailed role remains unclear [11].

In this study, we found that miR-25 was increased in OC tissues and cell lines. Inhibition of miR-25 led to a reduction of cell growth and motility. We further identified large tumor suppressor 2 (LATS2), a tumor suppressor, to be a direct target of miR-25in OC, and miR-25 functioned as an oncogene partially by targeting LATS2.

Materials and methods

OC tissues, cell lines, and transfection

18 paired malignant OC tissues and normal ovarian tissues were collected in the First Affiliated Hospital of Harbin Medical University. Written informed consent was obtained from each patient, and this work was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University. Collected tissues were immediately frozen in liquid nitrogen and stored at –80°C before RNA isolation.

Human ovarian cancer cells (OVCAR3, SKOV3, ES-2) and the human normal ovarian epithelial cell line (HOSE) were obtained from ATCC. Cells were maintained in RPMI-1640 medium supplemented with 10% FBS at 37 °C in a humidified chamber containing 5% CO2. Cells were plated ~24 h prior to transfection. Transient transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

RNA isolation and quantitative real time PCR (qRT-PCR)

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA). MiRNAs were isolated from OC tissues and cell lines using All-in-One microRNA extraction kits and measured using All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia, Carlsbad, CA, USA). The qRT-PCR experiments were performed using SYBR Green Reagents (TaKaRa, Tokyo, Japan) on ABI Prism 7700 system (ABI, Foster City, CA, USA). LATS2 primer: sense 5′-TCCTGCCACGACTTATTC-3′, 5′-GTGCCCGATTCATTAGC-3′. GAPDH primer: sense 5′-GAAGGTGAAGGTCGGAGTC-3′, 5′-GAAGATGGTGATGGGATTTC-3′. Primers for miR-25 and U6 were obtained from GeneCopoeia (Carlsbad, CA, USA). Expression of miR-25 was normalized with U6, and LATS2 was normalized with GAPDH. The expression was quantified using the $2^{-\Delta\Delta Ct}$ method.

Plasmids and luciferase activity assay

MiR-25 and the control mimics or inhibitors were purchased from RiboBio (Guangzhou, China). The LATS2 cDNA clone was obtained from Origene (Rockville, MD, USA), and subsequently subcloned into pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). The 3′-UTR of LATS2 which contains potential binding sites of miR-25 was amplified

using the following primers: sense 5′-CCCTCGAGCATCGCTTTCAATAGGCT-3′, antisense 5′-TTGCGGCCGCACAGCCACATCATCACCT-3′. The PCR product was subcloned into psiCHECK2 vector (Promega, Madison, WI, USA) within XhoI/NotI restriction sites. Mutant was created by mutating the seed regions of the miR-25 binding sites via a fast mutation kit (NEB, Ipswich, Canada).

For the luciferase activity assay, HEK293 cells were grown in 24-well plates and co-transfected with miR-25 or control (miR-NC) mimics and wild type (WT) or mutated (Mut) psi-Check2-LATS2-3′-UTR. Luciferase activity was examined 48 h after transfection using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) with an LB 960 Centro XS3 luminometer (Molecular Devices, Sunnyvale, CA, USA). Renilla luciferase activity was normalized to firefly luciferase activity.

Proliferation assays

5000 SKOV3 cells were seeded in 96-well plates and transfected with 100nM mimics, further incubated for 72 h following transfection. Cell proliferation was examined every 24 h using a CCK-8 assay kit (Beyotime, Shanghai, China).

In vitro migration and invasion assays

Cell migration and invasion assays were determined using transwell insert chambers. 1×105 transfected SKOV3 cells were resuspended in 200-μl RPMI-1640 medium, and placed into the upper chamber with or without Matrigel. RPMI-1640 with 10% FBS was added to the lower chamber as the chemoattractant. Cells were incubated for 24, cells remaining on the upper surface of membrane were carefully removed. Cells which migrated or invaded through the membrane were fixed with 4% polyoxymethylene and stained with 0.2% crystal violet, imaged and counted under an inverted microscope (Olympus, Tokyo, Japan).

Western blot

Proteins were extracted using RIPA buffer (Beyotime, Shanghai, China) with protease inhibitors. Equal amounts of protein samples were separated by 10% SDS-PAGE, and then electrotransferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocking, the membranes were immunoblotted overnight at 4 °C with primary antibodies, followed by HRP-conjugated secondary antibodies at 37°C for 1 h. Signals were detected using an ECL system. The intensity was determined using Image J software.

Statistical analysis

Data were expressed as mean ± SD. The differences were analyzed using two-sided Student t test or ANOVA using SPSS 16.0 software. Mann Whitney test was used in statistical analysis of tissue samples. P< 0.05 was considered statistically significant.

Results and discussion

1. miR-25 was increased in OC tissues and cell lines

To determine the expression of miR-25 in OC, 18 paired human OC and normal ovarian tissues were subjected to qRT-PCR. The expression of miR-25 was elevated in OC tissues compared to the normal ovarian counterparts (Fig. 1A). Furthermore, the expression of miR-25 in OC cell lines was also detected. The expression of miR-25 was elevated in three OC cell lines (OVCAR3, SKOV3, ES-2), compared with the human normal ovarian epithelial cell line, HOSE (Fig. 1B).

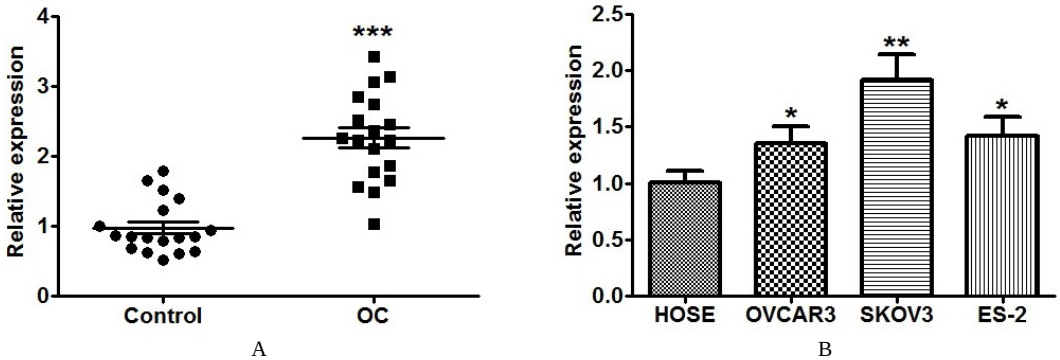

Fig. 1. miR-25 increased in OC tissues and cell lines: A - relative expression levels of miR-25 in 18 paired human OC and normal ovarian tissues measured by qRT-PCR; B - relative expression levels of miR-25 in three OC cell lines (OVCAR3, SKOV3, ES-2) and the human normal ovarian epithelial cell line (HOSE) were measured by qRT-PCR. U6 was used as an internal control.*P<0.05, **P<0.01, ***P<0.001 compared with control group

2. Inhibition of miR-25 suppressed proliferation and motility of OC cells

We further investigated the effects of miR-25 on proliferation and motility in OC cells. SKOV3 cells were transfected with miR-25 or control inhibitors (anti-miR-25, anti-miR-NC respectively) (Fig. 2A). CCK-8 assay was used to determine proliferation of SKOV3 cells, and the results showed that inhibition of miR-25 expression by inhibitors significantly suppressed prolif-

eration of SKOV3 cells (Fig. 2B). Similarly, in vitro migration and invasion assays found that inhibition of miR-25 substantially suppressed the motility abilities of SKOV3 cells (Fig. 2C, D). These data suggest that inhibition of miR-25 suppressed proliferation and motility of SKOV3 cells.

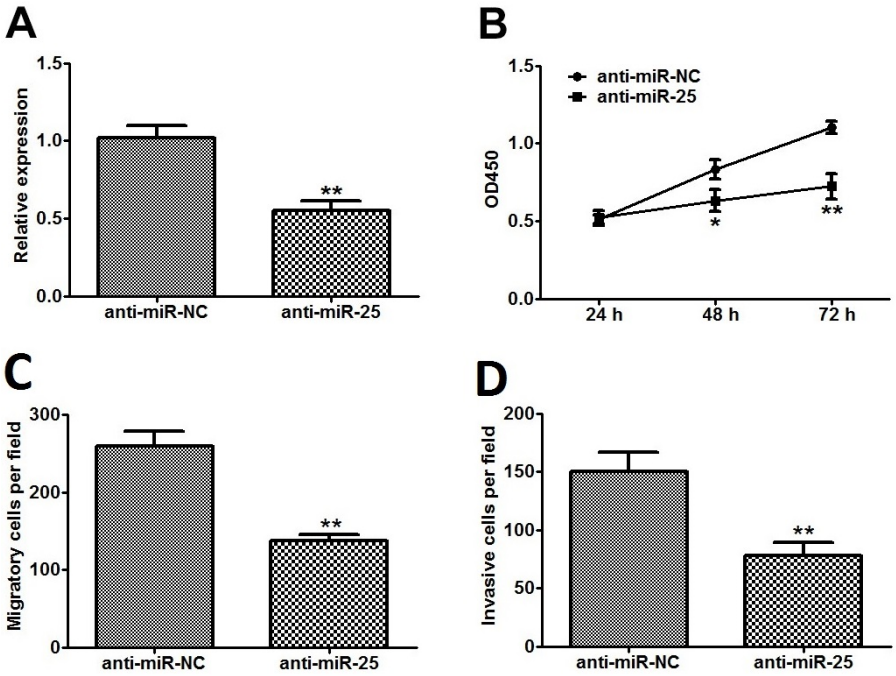

Fig. 2. Inhibition of miR-25 suppressed proliferation and motility of OC cells: A – SKOV3 cells were transfected with miR-25 or control inhibitors (anti-miR-25, anti-miR-NC respectively), and the expression of miR-25 was measured by qRT-PCR; B – CCK-8 assay was performed to examine proliferation of SKOV3 cells at different time points; C - in vitro migration and D - invasion assays were used to determine the motility abilities of SKOV3 cells. Experiments were performed in triplicate. *P<0.05, **P<0.01 compared with control group

3. LATS2 was a direct target of miR-25 in OC cells

Bioinformatics analysis using TargetScan 6.2 showed that LATS2 contains a potential binding sites of miR-25 (Fig. 3A). To confirm LATS2 as a target and regulated by miR-25 in OC cells, wild type (WT) LATS2 3′-UTR and the mutant (Mut) were cloned into luciferase reporter vectors, and luciferase activity assay was performed. The results showed that miR-25 significantly suppressed WT but not Mut luciferase activity (Fig. 3B). Furthermore, overexpression of miR-25 significantly reduced LATS2 protein level in SKOV3 cells (Fig. 3C). Together, these results suggest that LATS2 is a target of miR-25 in OC cells.

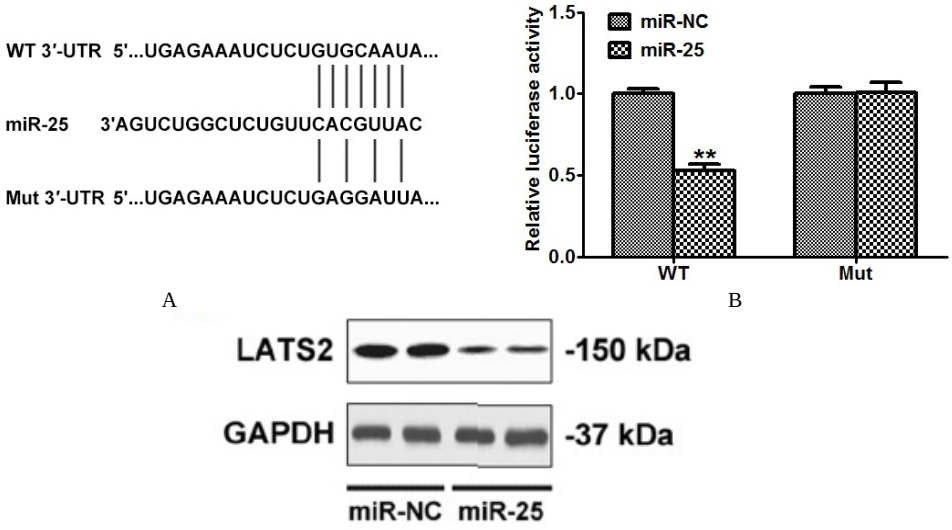

Fig. 3. LATS2 was a direct target of miR-25 in OC cells: A - the putative binding sequences of miR-25 in the 3′-UTR of LATS2; B – HEK293 cells were co-transfected with miR-25 or miR-NC with wild type (WT) or mutated (Mut) 3′-UTR of LATS2. Relative luciferase activity was assayed; C – SKOV3 cells transfected with miR-25 or miR-NC, and Western blot was used to detect the protein level of LATS2. GAPDH was used as control. Experiments were performed in triplicate. **P<0.01 compared with control group

4. miR-25 acted as an oncogene by targeting LATS2

We next tested whether restoration of LATS2 could reverse the oncogenic effects of miR-25 on OC cells. CCK-8, in vitro migration and invasion assays all showed that restoration of LATS2 significantly reversed the oncogenic effects of miR-25 on SKOV3 cells (Fig. 4A-C). The effect of pcDNA-LATS2 was determined by qRT-PCR (Fig. 4D). These data suggest that miR-25 acted as an oncogene by targeting LATS2.

In this study, we showed that miR-25 was increased in OC tissues and cell lines. Inhibition of miR-25 markedly suppressed OC cell growth and motility. Moreover, we demonstrated that LATS2 was a direct target of miR-25 in OC cells, and restoration of LATS2 expression attenuated the oncogenic function of miR-25 in OC cells. These findings suggest that miR-25 may function as a novel oncogene in OC and contribute to tumor progression of OC.

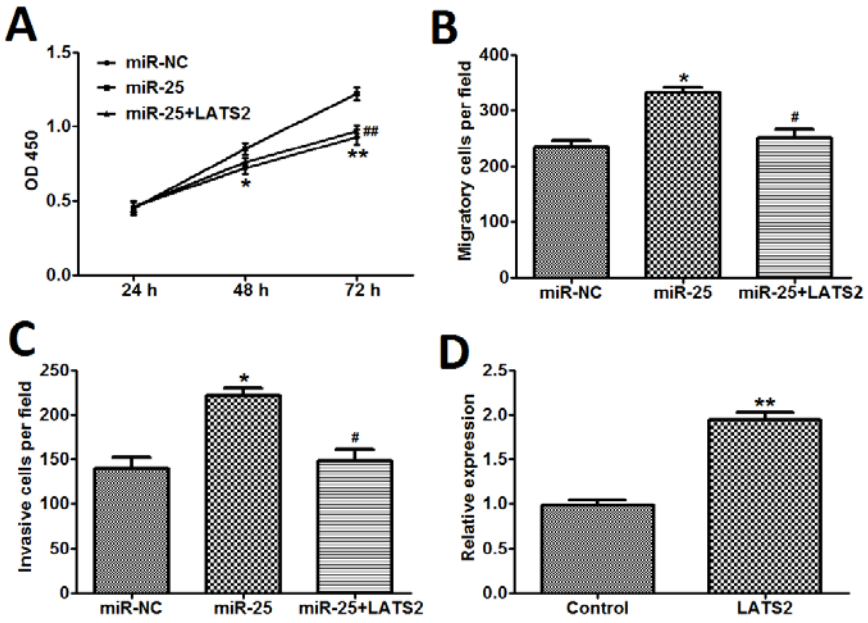

Fig. 4. miR-25 acted as an oncogene by targeting LATS2: A – SKOV3 cells were co-transfected with miR-25 and pcDNA-LATS2 or the vector, CCK-8 assay was performed to examine proliferation of SKOV3 cells at different time points; B - In vitro migration and C - invasion assays were used to determine the motility abilities of SKOV3 cells; D – SKOV3 cells transfected with pcDNA-LATS2 or vector, and qRT-PCR was used to detect the mRNA level of LATS2. GAPDH was used as control. Experiments were performed in triplicate. *P<0.05, **P<0.01 compared with control group. #P<0.05, ##P<0.01 compared with miR-25 group

Increasing evidence has revealed that miRNAs are key regulators of protein coding genes involved in various cancers, including human OC. Recent studies showed a direct link between miRNAs and OC. For instance, Pecot CV et al. reported that miR-200 inhibited angiogenesis and induced vascular normalization in several tumors through direct and indirect mechanisms partially via targeting interleukin-8 [12]. Wang S et al. showed that elevation of miR-203 was associated with advanced progression and poor prognosis in EOC [13]. Here we showed that miR-25 played an oncogenic role in OC. MiR-25 belongs to the miR-92a family, which includes miR-25, miR-92a-1/2, and miR-363. Aberrant expression of miR-92a family was found in multiple cancers, and the dysregulation of miR-92a family was associated with tumorigenesis and tumor development [14]. Zhao H et al. reported that miR-25 was increased in gastric cancer, and suppressed gastric cancer development and progression by targeting reversion-inducing-cysteine-rich protein with kazal motifs (RECK) [15]. Xu X et al. found that miR-25 promoted migration and invasion of esophageal squamous cell carcinoma cells [16]. In OC, Wang X et al. reported that miR-25 was increased in OC, and elevated expression of miR-25 was associated with poor prognosis of EOC [11]. Zhang H et al. also found that miR-25 promoted OC cell proliferation by targeting B-cell lymphoma 2 interacting mediator of cell death (Bim) [17]. Our data further explored the oncogenic role of miR-25 in OC via targeting LATS2.

LATS2 is a member of the LATS tumor suppressor family, and is located in human chromosome 13q11-12 [18]. The LATS family plays an essential role in mediating Hippo (Hpo) growth inhibitory signaling [19]. LATS2 is involved in a variety of cellular processes, including proliferation, angiogenesis, apoptosis, migration and invasion [20-22]. For example, Dai X et

al. reported that LATS1/2 phosphorylated angiomotin suppressed F-actin binding, cell migration, and angiogenesis [21]. LATS2 has been reported to be decreased, and act as a tumor suppressor in various cancers, including breast cancer, lung cancer, and hepatocellular carcinoma [23-25]. LATS2 has been found to be regulated by many miRNAs, including miR-93, miR-181b, and miR-195 [24-26]. Here, we showed that miR-25 promoted OC cell growth and motility partially by targeting LATS2.

Taken together, our data provide novel evidence that miR-25 negatively regulated LATS2 expression and promoted OC cell growth and motility, suggesting that the overexpression of miR-25 may be a potential therapeutic biomarker for OC and provide a potential therapeutic strategy for OC treatment.

Authors:

Shujun Feng – Professor of Obstetrics and Gynecology Department, the Second Affiliated Hospital of Harbin Medical University. Address: Harbin, Heilongjiang 150000, China.

Wenjing Pan – Professor of Obstetrics and Gynecology Department, the Second Affiliated Hospital of Harbin Medical University. Address: Harbin, Heilongjiang 150000, China.

Ye Jin – PhD student of Obstetrics and Gynecology Department, the First Affiliated Hospital of Harbin Medical University. Address: Harbin, Heilongjiang 150001, China.

Elena S. Kapora – assistant of Department of obstetrics and gynecology №1 of Bashkir State Medical University, PhD student of Obstetrics and Gynecology Department, the Second Affiliated Hospital of Harbin Medical University. Address: Harbin, Heilongjiang 150000, China.

Jianhua Zheng – Professor, Chief of Obstetrics and Gynecology Department, the First Affiliated Hospital of Harbin Medical University. Address: Harbin, Heilongjiang 150001, China. E-mail: jhua_zheng@163.com.

Indira V. Sakhautdinova – Doctor of medical Sciences, Professor, Chief of the Department of obstetrics and gynecology №1 of Bashkir State Medical University. Address: 450008, Ufa, Lenina str. 3. E-mail: bgmu.ag@yandex.ru.

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